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Amelogenin in Cranio-facial Development: The Tooth as a Model to Study the Role of Amelogenin During Embryogenesis

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ABSTRACT The amelogenins comprise 90% of the developing extracellular enamel matrix proteins and play a major role in the biomineralization and structural organization of enamel. Amelogenins were also detected, in smaller amounts, in postnatal calcifying mesenchymal tissues, and in several nonmineralizing tissues including brain. Low molecular mass amelogenin isoforms were suggested to have signaling activity; to produce ectopically chondrogenic and osteogenic-like tissue and to affect mouse tooth germ differentiation in vitro. Recently, some amelogenin isoforms were found to bind to the cell surface receptors; LAMP-1, LAMP-2 and CD63, and subsequently localize to the perinuclear region of the cell. The recombinant amelogenin protein ($rHAM^{+}$) alone brought about regeneration of the tooth supporting tissues: cementum, periodontal ligament and alveolar bone, in the dog model, through recruitment of progenitor cells and mesenchymal stem cells.

We show that amelogenin is expressed in various tissues of the developing mouse embryonic cranio-facial complex such as brain, eye, ganglia, peripheral nerve trunks, cartilage and bone, and is already expressed at E10.5 in the brain and eye, long before the initiation of tooth formation. Amelogenin protein expression was detected in the tooth germ (dental lamina) already at E13.5, much earlier than previously reported (E19). Application of amelogenin ($rHAM^{+}$) beads together with DiI, on E13.5 and E14.5 embryonic mandibular mesenchyme and on embryonic tooth germ, revealed recruitment of mesenchymal cells. The present results indicate that amelogenin has an important role in many tissues of the cranio-facial complex during mouse embryonic development and differentiation, and might be a multifunctional protein. *J. Exp. Zool. (Mol. Dev. Evol.) 310B, 2008.* © 2008 Wiley-Liss, Inc.

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The amelogenins, which comprise about 90% of the enamel matrix proteins (Termine et al., '80), play a major role in the biomineralization and structural organization of enamel (Robinson et al., '88; Fincham et al., '94). Amelogenins are hydrophobic molecules that self-assemble in vitro and in vivo into nanospheric structures, which regulate

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the oriented and elongated growth, shape and size of the enamel mineral crystal (Fincham et al., '94; Du et al., 2005; Veis, 2005). During enamel development and mineralization, the amelogenins in the extracellular enamel are sequentially and discretely degraded by specific proteases the metalloprotease Enamelysin (MMP20) and the serine protease KLK4 (Simmer and Hu, 2002). The amelogenins are eventually, together with other enamel matrix proteins, replaced by mineral ions calcium and phosphorus, the enamel finally becoming hard, fully mineralized (96%) and mature (Termine et al., '80; Deutsch et al., '95; Robinson et al., '98).

The mouse amelogenin gene is mapped to the X chromosome (Lau et al., '89; Fincham et al., '91), whereas in the human it maps to Xp22.1–p22.3 and Yp11.2 chromosomes (Lau et al., '89; Salido et al., '92). The gene contains nine exons (Li et al., '98; Baba et al., 2002) that undergo extensive alternative mRNA splicing (Simmer and Fincham, '95; Veis, 2003; Papagerakis et al., 2005). The most abundant amelogenin isoform in the mouse enamel is M180 that is encoded by exons 1–7, skipping exon 4.

In human, mutations in the X-chromosomal copy of the amelogenin gene *AMELX* have been associated with the hereditary disease Amelogenesis Imperfecta (Hart et al., 2000). Defective enamel formation has also been demonstrated by knockdown of amelogenin expression using antisense oligonucleotides (Diekwisch et al., '93), ribozymes (Lyngstadaas et al., '95) and in amelogenin knockout and transgenic mice, which resulted in enamel characteristic of hypoplastic amelogenesis imperfecta, with reduced enamel thickness (Gibson et al., 2001).

For decades amelogenin was thought to be exclusively an enamel (epithelial origin) protein. However, in more recent years different isoforms of amelogenin have also been found in the dentin matrix (Hammarstrom et al., '97; Nebgen et al., '99) and the odontoblasts (Oida et al., 2002; Papagerakis et al., 2003), during cementogenesis in remnants of Hertwig's root sheath and in periodontal ligament (PDL) cells (Fong and Hammarstrom, 2000; Janones et al., 2005). Very recently, we have described amelogenin expression in long bone cells; osteocytes, osteoblasts and osteoclasts, and some of the bone marrow cells. Amelogenin is also expressed in chondrocytes of the articular cartilage and differentially in cell layers of the epiphyseal growth plate. We have identified amelogenin expression in long bone marrow cells, some of which are mesenchymal stem cells, and in cells surrounding blood vessels (Haze et al., 2007). Amelogenin expression was also identified in cells of nonmineralizing tissues such as the brain, specifically in the glial cells, in salivary glands and in some of the hematopoietic cells such as megakaryocytes and macrophage (Deutsch et al., 2006; Haze et al., 2007). The relatively large number of amelogenin alternatively spliced mRNA translated polypeptides and the fact that amelogenin is expressed in different tissues (calcifying and soft tissues) and of different embryonic origin, possibly reflect different functions of amelogenin.

Low molecular mass amelogenin isoforms were suggested to be signal molecules; were shown to produce ectopically chondrogenic and osteogenic-like tissue and to have different signaling effects on ameloblasts and odontoblasts differentiation in developing tooth culture model, and when implanted in the tooth pulp (Nebgen et al., '99; Veis, 2003; Lacerda-Pinheiro et al., 2006a,b; Zeichner-David et al., 2006; Jegat et al., 2007). Amelogenin (M180), the amelogenin isoform LRAP (leucin-rich amelogenin peptide), and some of the amelogenin degradation products, were found to bind to the cell surface receptors; LAMP-1, LAMP-2 and CD63, which are ubiquitously expressed lysosomal integral membrane proteins that are also localized to the plasma membrane. Shapiro et al. found that exogenously added amelogenin moves rapidly into established LAMP-1 positive vesicles that subsequently localize to the perinuclear region of the cell (Shapiro et al., 2007). Zou et al. reported the exact regions and sequences that bind amelogenin to these receptors (Zou et al., 2007).

A major discovery that highlights a new role for enamel matrix proteins was the finding that the application of an enamel matrix protein extract to tooth root surfaces in sites of diseased periodontium promotes the regeneration of all the periodontal tissues (Hammarstrom et al., '97). It was therefore suggested that amelogenin is responsible for this regeneration. Recently, we showed that the recombinant human amelogenin protein ($rHAM^{+}$), produced in the eukaryotic *baculovirus* system (Taylor et al., 2006), causes significant and progressive regeneration of all three tooth supporting tissues; alveolar bone, PDL and cementum, after induction of chronic periodontitis, in the dog. Further immunohistochemistry studies, using markers for mesenchymal stem cells, combined with the above findings, suggested that amelogenin induces, directly or

indirectly, recruitment of mesenchymal stem cells and/or progenitor cells, during the regeneration of the tooth supporting tissues (Deutsch et al., 2006).

Large amount of information is available in the literature on genes and their corresponding proteins, associated with embryonic tooth development, morphogenesis and differentiation, such as signaling molecules, growth factors, homeoboxes etc. (Tucker and Sharpe, '99; Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004; Hu et al., 2006; Mitsiadis and Smith, 2006). The same is true for all other cranio-facial organs. However, almost no data is available on amelogenin expression and function in the early stages of embryonic cranio-facial development.

In this study we focused on the spatio-temporal expression of amelogenin in different tissues of the developing embryonic mouse cranio-facial complex, such as the tooth germ, brain, eye, ganglia, peripheral nerve trunks, cartilage and bone. Our results indicate that amelogenin is expressed in many tissues of the cranio-facial complex during mouse embryonic development and differentiation, pointing to the possibility that it might be a multifunctional protein.

RESULTS

Amelogenin mRNA expression in the cranio-facial complex during mouse embryonic days E10.5–E17.5

The expression of amelogenin mRNA, in the cranio-facial complex at E10.5 up to E17.5, was analyzed by RT-PCR, followed by cDNA sequencing (Fig. 1A). The most abundant amelogenin isoform in the extracellular enamel matrix, M180 (exons 1–7 lacking exon 4, Fig. 1B), was detected along the different stages of mouse embryonic cranio-facial development. Other amelogenin isoforms might also be present. We are currently looking for such isoforms.

Spatio-temporal expression of amelogenin in the cranio-facial complex during E10.5-E18.5 mouse embryonic development (Fig. 2)

Amelogenin expression (red brown staining) was detected in different tissues of the mouse embryonic cranio-facial complex, at different stages of development. No staining was observed in the corresponding control (PBS) sections (not shown).

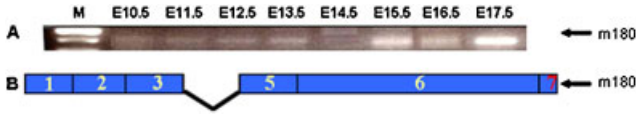

Fig. 1. (**A**) Expression of amelogenin cDNA isoform M180 in the mouse embryonic cranio-facial complex at E10.5 through E17.5. (**B**) Schematic representation of the exon structure of M180.

Amelogenin expression in the developing eye (Fig. 2a)

At E10.5 positive staining for amelogenin was detected at the outer peripheral area of the inner part of the optic cup (OC), which gives rise to the photoreceptors. At E11.5 staining in the area of the photoreceptors (Pr) facing the future pigment epithelium region, was more intense. At E12.5, faint staining was also detected in fibers at the inner side of the optic nerve, with the precursors of the photoreceptors (Pr) being more intensely stained. Cells at the periphery of the lens stained positively, as well as the lens fibers. In the epithelium that will develop into the cornea few cells were positively stained (Cr). At E13.5, the fibers, connective tissue and choroidal blood vessels (Ch) were positively stained, while still few cells in the cornea (Cr) were stained. At E14.5 lens fibers (LF) were positively stained, and faint staining was detected in most of the cornea (Cr) cells, as well as in the sclera (Sc). Staining was also observed in the vitreous (Vi) but not in the retina (Re), as the photoreceptors loose their staining. At E15.5 the lining epithelium at the anterior part of the lens, as well as the retina (Re), did not stain, whereas the choroidal blood vessels stained positively (Ch). At E16.5 mainly lens fibers (LF) and the entire choroid (Ch) was positively stained. In the ganglion layer (GC) of the retina (Re), cell bodies (cytoplasm) and fibers were stained, but at that stage the bipolar cell layer, the photoreceptors and the pigment epithelium did not stain with amelogenin antibodies. At E18.5 the lens (LF) was completely stained. The retinal vessels and the sub-epithelial areas (EI) over the eyelids were also stained

Amelogenin expression in the developing brain (Fig. 2a)

At E10.5 positive staining for amelogenin was detected in the cranial part of the neural tube at the external limiting membrane (ELM), where glial cells were stained. When fibers were detected—they apparently also stained for

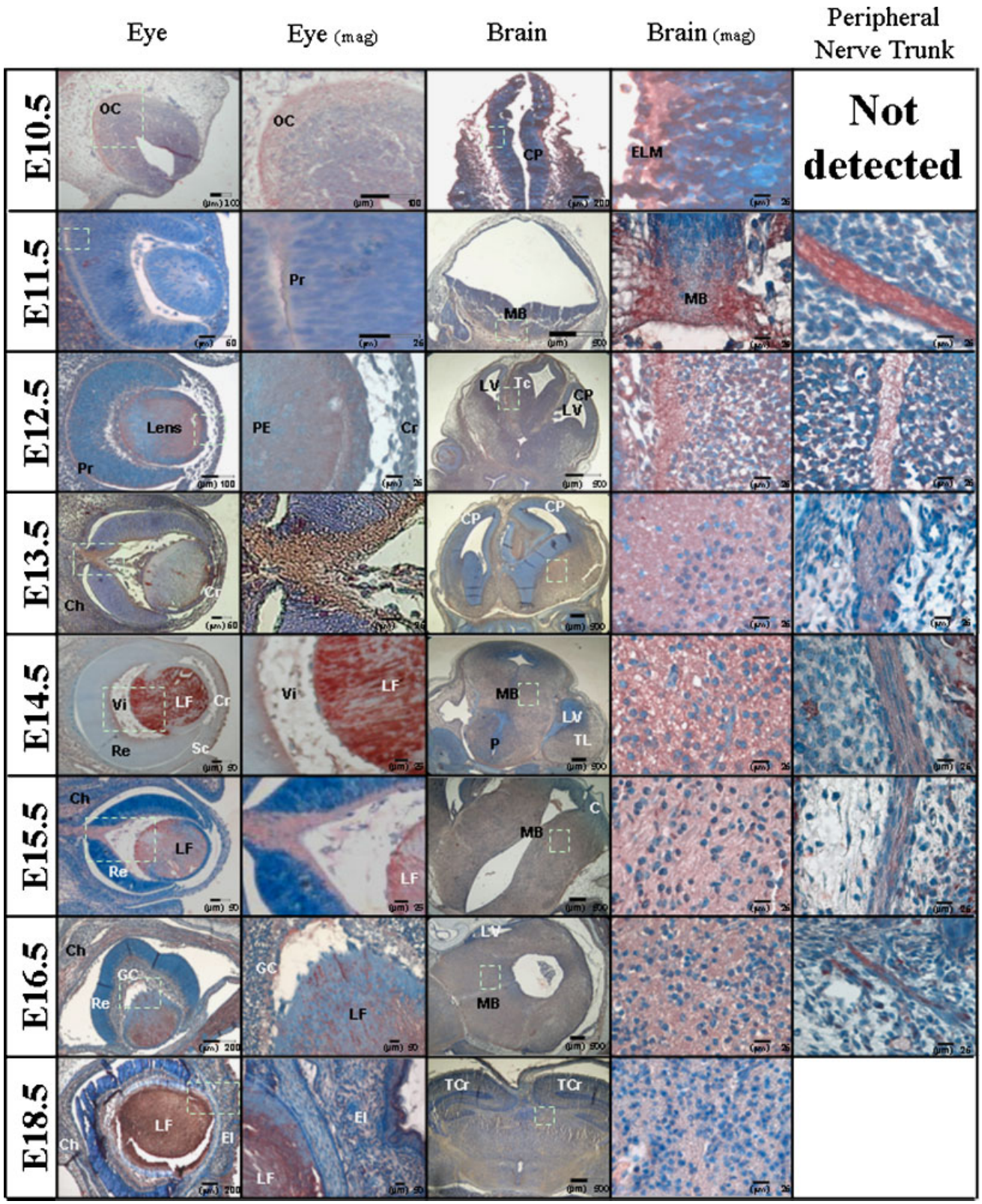

Fig. 2. *Contined.*

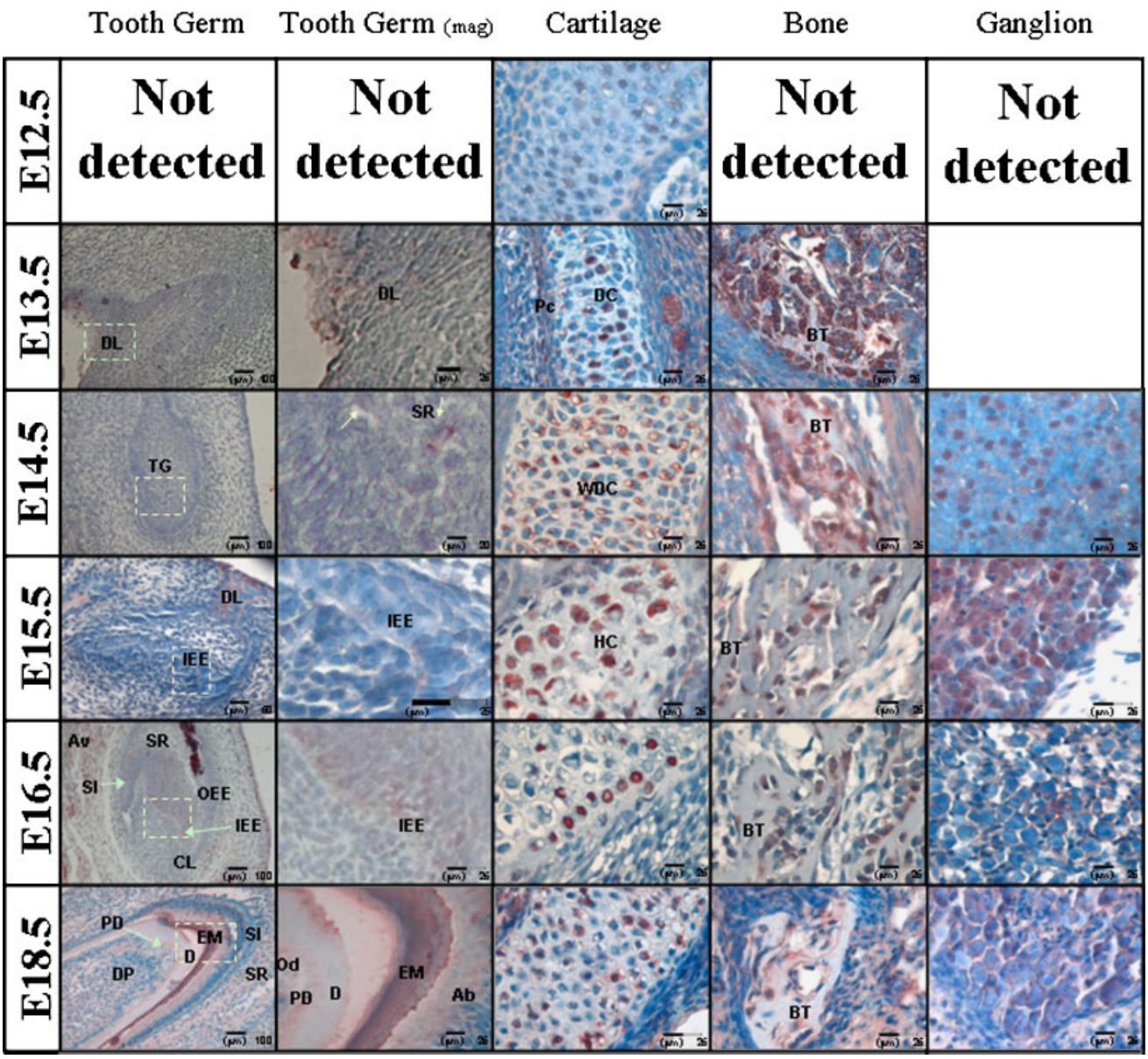

Fig. 2. Spatio-temporal distribution of amelogenin expression in tissues of E10.5–E18.5 mouse embryonic cranio-facial complex (Amelogenin expression—brown red staining, hematoxylin—blue staining). Eye: Ch—choroidal blood vessels; Cr—cornea; EI—sub-epithelial region; GC—ganglion layer; LF—lens fibers; OC—optic cup; Pr—photoreceptors; Re—retina; Sc—sclera; Vi—vitreous. Brain: C—cortex; CP—cortical plate; ELM—external limiting membrane; LV—lateral ventricle; MB—midbrain; P—pons; Tc—telencephalon; TCr—temporal cortex TL—temporal lobe. Peripheral nerve trunk: NF—peripheral nerve fibers. Tooth germ: Ab—ameloblasts; Av—alveolar bone trabecules; CL—cervical loop; D—dentin; DL—dental lamina; DP—dental papilla; EM—extracellular enamel matrix; IEE—inner enamel epithelium; Od—odontoblasts; OEE—outer enamel epithelium; PD—pre-dentin; SI—stratum intermedium; SR—stellate reticulum; TG—tooth germ. Cartilage: DC—differentiated chondrocytes; HC—Hypertrophic cartilage; Pc—Pre-chondrocytes; WDC—well developed chondrocytes. Bone: BT—bone trabecules. (**A**) In the eye, brain and peripheral nerve trunk at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, E16.5 and E18.5. (**B**) In the tooth germ, cartilage, bone and ganglion at E12.5, E13.5, E14.5, E15.5, E16.5 and E18.5. No amelogenin staining was detected in the corresponding control (PBS) sections (not shown).

amelogenin. Most of the staining in the nerve fibers was probably associated with the glial cells, however, it is not clear whether some axons or dendrites were also stained. At E11.5 amelogenin expression was detected in the anterior (ventral) regions of the midbrain (MB), mainly in fiber-like structures and glial cells. Staining was also observed in the tissue that will eventually develop into cartilage at the base of the skull. At E12.5 positive, but weak, amelogenin staining was

detected in the medial part (that is rich in fibers) of the telencephalon (Tc), whereas no staining was detected in the cortical plate (CP), which, at that stage comprised mainly of neuroblasts. In general, amelogenin staining was observed in both brain hemispheres in fiber-like structures. In most cases, the glial cells stained positively, but other cell types (neurons) might also be involved. At E13.5, fibers (white material under the cortical plate) stained positively whereas no staining was detected in the cortical neurons. At E14.5 dispersed positive staining of glial cells throughout the brain especially in the midbrain (MB) and pons (P), and not only in fiber-like structures, was detected. At E15.5, staining of nerve fibers in the mid-brain (MB) was less prominent, but faint staining in the cerebral cortex (CP) was now demonstrated. At E16.5 and E18.5, staining was detected throughout the brain except for a thin layer of cortical neurons and around the lateral ventricle that was negative (LV). Stronger staining was detected at regions rich in fibers or where neurons were not dense; this staining was detected in glial cells. However it is unclear whether neurons, axons and dendrites were also stained.

Amelogenin expression in the developing peripheral nerve tracts and fibers (Fig. 2a)

At E10.5 almost no nerve fibers were detected. At E11.5–E16.5 amelogenin expression was detected in the developing peripheral nerve (NF), most probably in the glial cells. It is unclear whether axons and/or dendrites were also stained.

Amelogenin expression in the developing ganglia and cervical spinal cord (Fig. 2b)

At E10.5 the neural tube was not stained for amelogenin. At E11.5 amelogenin expression was not detected in the trigeminal ganglion, or in the spinal cord. At E12.5 amelogenin staining was observed in fiber-like structures in the ventral part of the spinal cord. In sites of the spinal cord where vertebrae were more organized less amelogenin staining was detected. At E13.5 positive staining was detected in dorsal spinal cord ganglia, however, it is not yet clear which type of cells were stained. At E14.5 the entire spinal cord stained positively for amelogenin; the staining was further intensified at E15.5. From E14.5 and thereafter up to E18.5 the trigeminal ganglion and ganglia at the vicinity of the tooth germ stained positively.

Amelogenin expression in the developing tooth germ (Fig. 2b)

At E13.5 cells in the dental lamina (DL) were positively stained. At E14.5, in the tooth germ (TG), specifically in the region of the stellate reticulum (SR) faint positive staining was also detected. At E15.5 staining was still detected in the dental lamina (DL), and was also detected in the inner enamel epithelium (IEE). At E16.5, staining was observed in the inner enamel epithelium (IEE) and stratum intermedium (SI). Faint staining was detected in the stellate reticulum (SR) and almost no staining in the outer enamel epithelium (OEE). The oral epithelium was also stained (not shown) whereas the cervical loop (CL) was faintly stained. Amelogenin staining was also detected in developing alveolar bone trabeculae (Av). At E18.5 the dental papilla (DP), odontoblasts (Od) and pre-dentin (PD) were slightly stained, the dentin (D) did not seem to be stained, followed by very strong purple brown staining of the developing extracellular enamel matrix (EM). The ameloblasts (Ab) were stained, but not at the outmost region of the ameloblast's cell nucleus. The stratum intermedium (SI) and stellate reticulum (SR) surrounding the ameloblasts were only slightly stained. These layers are covered by the outer enamel epithelium that was also positively stained.

Amelogenin expression in the developing cartilage (Fig. 2b)

At E11.5, before cartilage was detected, amelogenin staining was observed in the tissue that will eventually develop into cartilage (Pc—prechondrocytes) at the base of the skull. At E12.5 some staining was detected in the developing cartilage, mainly in surrounding blood vessels but also in some chondrocytes. At E13.5 the cartilage of the vertebrae alongside the cervical spinal cord stained positively. The chondrocytes that were not fully differentiated did not stain, whereas differentiated chondrocytes (DC) that underwent hypertrophy stained positively for amelogenin. At E14.5, prehypertrophic and hypertrophic chondrocytes (WDC) and the cartilage beneath the perichondrium stained positively. This was also observed at E15.5 and E16.5, as hypertrophic cartilage (HC) and perichondrium, as well as blood vessels in various cranio-facial regions stained positively for amelogenin. At E18.5 both chondrocytes and blood vessels in the hypertrophic cartilage stained positively.

Amelogenin expression in the developing bone (Fig. 2b)

Bone in the cranio-facial region was first detected at E13.5. At that developmental stage periosteal osteoblasts, at the beginning of bone organization, stained positively. At E14.5, most cell types and not just osteoblasts in the cranial bones (mainly membranous bone that does not develop from cartilage), were positively stained. The mandibular alveolar bone, which is composed of periosteal bone, stained positively at E14.5 and E15.5. However, only faint staining was detected in the endochondral mandibular condyle. At E16.5, both endochondral and membranous bones in the mandible, maxilla and their surrounding periosteum, stained for amelogenin. This positive staining was also observed at E18.5.

Amelogenin expression in additional developing tissues (not shown)

From E10.5 through E18.5 the mesenchyme and connective tissue were positively stained for amelogenin. On E11.5 amelogenin expression was detected in loosely packed mesenchymal cells at the base of the skull. Amelogenin staining at E11.5 was also detected at the region of both, yet vertical, palatine shelves. The palate continued to be positive at least until E15.5. The skin and the connective tissue just beneath the skin epithelium (future dermis) did not stain at E11.5–E16.5. Only at E18.5 the dermis stained positively for amelogenin, whereas the epithelium still did not stain. At E13.5 the connective tissue surrounding the brain (brain membranes) stained positively. At E15.5 amelogenin staining was detected in the salivary glands, which apparently disappeared at E18.5.

Recruitment of mesenchymal cells by amelogenin in the developing mandible

Agarose beads containing the recombinant human amelogenin ($rHAM^+$, long black arrow) (Taylor et al., 2006), were placed along different regions of the mandibular mesenchyme of E13.5 mouse embryos. The experimental beads were on one side of the mandible, whereas the control beads (long white arrow) were on the contra-lateral side. The ex-vivo mandible was placed in culture for one day and then coronal sections were made. Mesenchymal cells apparently moved toward the $rHAM^+$ beads and surround the beads (short arrows), whereas no such cell recruitment could be seen around the control PBS agarose bead (Fig. 3).

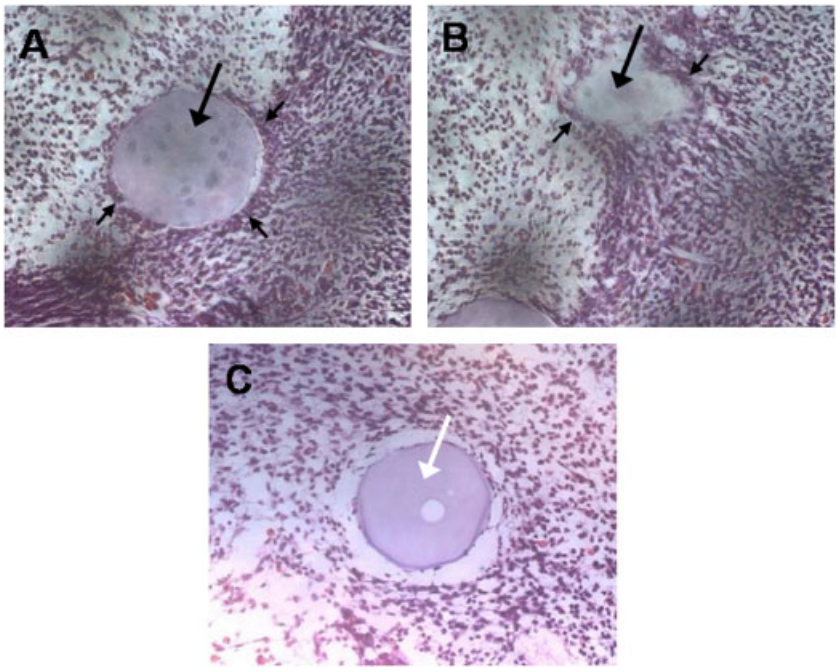

Fig. 3. Possible recruitment of mesenchymal cells in the developing mandible by amelogenin ($rHAM^+$) beads but not by control PBS beads. (**A**, **B**) $rHAM^+$ beads (long black arrow) one day after bead application on E13.5 mouse embryonic mandibles. Note the ring of cells surrounding the $rHAM^+$ beads (short arrows). (**C**) PBS bead (long white arrow). No such phenomenon is detected around the control bead.

Recruitment of mesenchymal cells by amelogenin in the developing incisor tooth germ

Agarose beads containing rHAM$^+$ or PBS (control) were placed on the mesenchymal region of the incisor tooth germ of 250 μm frontal sections of E14.5 mandibles. Changes in DiI distribution between 0 and 3 days in culture showed movement (recruitment) of mesenchymal cells toward the rHAM$^+$ bead (Fig. 4A–D), which eventually partially covered the bead, whereas no such movement was observed toward the control PBS bead (Fig. 4E–H).

DISCUSSION

In this study we focused on the spatio-temporal expression of amelogenin in different tissues of the developing embryonic mouse cranio-facial complex. Our results indicate possible signaling and structural roles for amelogenin during early stages of embryonic cranio-facial development, in addition to its known function during enamel biomineralization. The results clearly show that the expression of amelogenin in different tissues is dynamic and depends on the embryonic developmental stage. The recruitment of mesenchymal cells in the tooth germ and mandible, at early stages of embryonic development, points to the functional significance of amelogenin in these tissues.

Many studies in the field of molecular biology and biochemistry, have described the function of amelogenin in structural organization and biomineralization of enamel; in controlling the size, shape and direction of formation of the enamel mineral crystallites (Fincham et al., '94; Du et al., 2005; Veis, 2005). In the past few years, cell signaling functions have been attributed to amelogenin low-mass isoforms such as LRAP (A-4/M59) (Nebgen et al., '99; Veis, 2003; Lacerda-Pinheiro et al., 2006a,b; Zeichner-David et al., 2006; Jegat et al., 2007). The cell surface receptors for amelogenin (M180 and LRAP), were identified, and the exact binding regions of amelogenin to its receptors was deciphered (Shapiro et al., 2007; Zou et al., 2007). We have recently shown that recombinant human amelogenin (equivalent to M180) brought about regeneration of the tooth supporting mesenchymal tissues: cementum, PDL and alveolar bone in the dog model. This regeneration process involved recruitment of

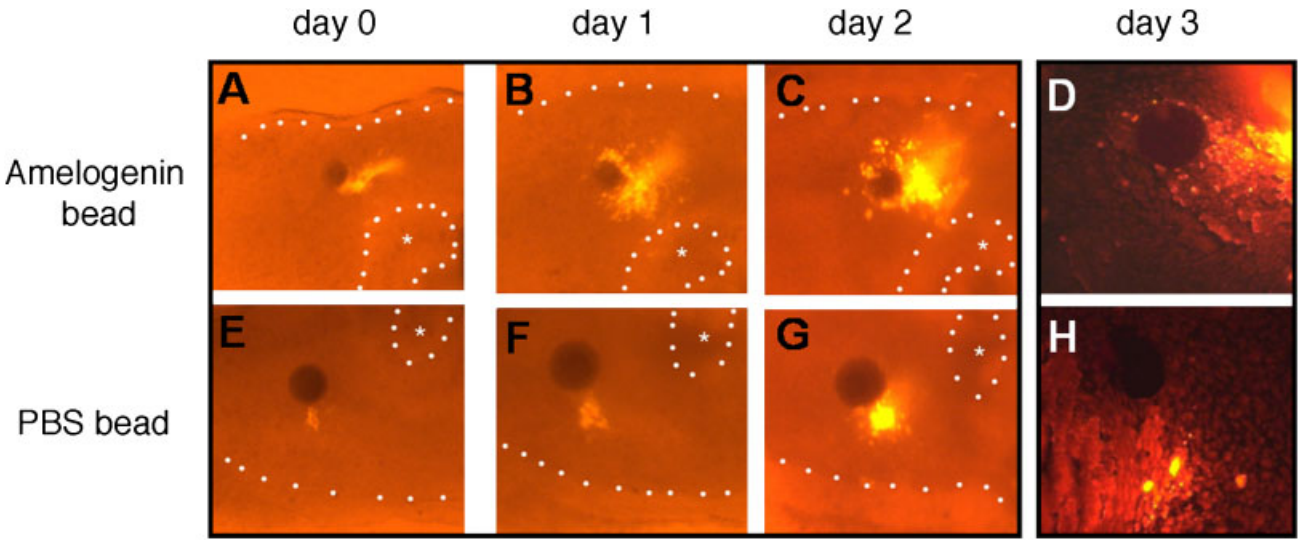

Fig. 4. Movement (recruitment) of DiI—labeled mesenchymal cells in the incisor tooth germ region, toward amelogenin (rHAM$^+$) beads but not toward control PBS bead. Frontal slices through the incisor region of E14.5 mandibles cultured for three days after addition of beads. Oral and incisor epithelium are outlined in white. The mesenchyme around the incisor cap stage tooth germs (outlined and marked by ∗) was labeled with DiI (orange). (A–D) rHAM$^+$ bead at day 0 (A), day 1 (B), day 2 (C), day 3 (D). (A–C) Merged light/dark field image of cultured slice showing DiI label moving toward and around the rHAM$^+$ bead. (D) Section showing DiI cells associated with the rHAM$^+$ bead. (E–H) Similar slice with a PBS (control) bead at day 0 (A), day 1 (B), day 2 (C), day 3 (D). (E–G) Merged light/dark field image of cultured slice showing DiI label remaining distinct from the PBS bead. (H) Section showing DiI cells not associated with the PBS bead.

mesenchymal stem cells, also indicating signaling role(s) for amelogenin (Haze. et al. in submission). All these studies were performed on postnatal tissues. Very few detailed studies were carried out on amelogenin expression in the cranio-facial complex during prenatal development, and these studies focused mainly on the late stages of prenatal development when ameloblasts and odontoblasts had already differentiated, and begun to produce developing enamel and the underlying dentin (Zeichner-David et al., '97; Lacerda-Pinheiro et al., 2006b). This is in contrast to the vast information in the literature on other genes, their corresponding proteins and the pathways associated with embryonic cranio-facial and specifically with tooth development, morphogenesis and differentiation (Tucker and Sharpe, '99; Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004; Mitsiadis and Smith, 2006).

In this study we showed that amelogenin mRNA (Fig. 1) and protein (Fig. 2) are expressed in the cranio-facial complex from early stages of development (E10.5). Amelogenin protein is expressed in some tissues of the cranio-facial complex long before the initiation of tooth formation. The amelogenin protein was mainly identified in glial cells, in neural-crest-derived cells and possibly (but it is still not clear yet) in neurons, as it was detected in the brain, the retina of the eye, peripheral ganglia and the peripheral nerve trunks. In the cranio-facial complex, neural crest cells also give rise to nonneuronal ecto-mesenchymal tissues such as bone, cartilage and mesenchymal regions of the teeth (Le Douarin et al., 2007) that were all positive for amelogenin. Interestingly, the strongest staining for amelogenin was detected in the ameloblasts, extracellular enamel matrix and the eye lens that are not thought to be neural crest derived. Other epithelial tissues were not intensely stained (cornea), or staining was detected at later stages of development (skin, eyelids).

The expression of amelogenin was studied during mouse embryonic tooth germ development, from early stages of development, when the information on the spatio-temporal expression of amelogenin is rather scarce, to E18.5, when amelogenin expression in the tooth germ (near birth) and postnatally is well documented. Amelogenin protein expression was seen already at E13.5 in the dental lamina, and continued through embryonic tooth development to E18.5. From E13.5 up to E16.5 (E17.5 was not analyzed), no extracellular enamel or dentin is yet formed, and no biomineralization of enamel or dentin takes place. Amelogenin, therefore, has different function(s) at these stages than that of regulating the size, shape and direction of mineral crystal growth. Late in development and close to birth (E18.5), amelogenin expression was mainly seen in the mineralizing extracellular enamel matrix and in the secreting ameloblasts, and also in the stratum intermedium, pre-dentin and dentin, and some expression in the dental papilla. The latter expression pattern, observed by us at E18.5, has been extensively described, mainly in postnatal mice.

A hint on amelogenin function in the early stages of tooth and mandible development emerges from the application of amelogenin beads on E13.5 mouse embryonic mandibular mesenchyme, which brought about recruitment and cell movement toward the beads (Fig. 3). This observation was strengthened by the application of amelogenin beads, in combination with DiI application, on E13.5 and E14.5 embryonic incisor and first molar (not shown) tooth germs mesenchymal tissue, respectively. The DiI, which binds to cell membranes, revealed movement of mesenchymal cells toward the amelogenin beads, partially covering the beads after 2 and 3 days (Fig. 4). The amelogenin beads contained the recombinant human amelogenin protein rHAM^{+} that is the human amelogenin isoform corresponding to the mouse isoform M180 (which contains exons 1–7, lacking exon 4). No such recruitment of mesenchymal cells was detected toward the control PBS bead. As amelogenin is endogenously expressed in these mesenchymal and tooth germ tissues at the days rHAM^{+} beads were applied (Fig. 2), these experiments represent over-expression and not ectopic expression. The observed cell recruitment suggests a signaling function for amelogenin. This is in line with the accumulating data on the signaling function of amelogenin, including our recent data (Haze et al. in submission). Other studies in which low molecular mass amelogenin isoforms produced ectopically chondrogenic and osteogenic-like tissue and caused ameloblasts and odontoblasts differentiation suggested prenatal and postnatal signaling effects for the different amelogenin isoforms (Nebgen et al., '99; Veis, 2003; Lacerda-Pinheiro et al., 2006a,b; Jegat et al., 2007) (also see introduction on possible signaling activity of amelogenin).

As was previously described, amelogenin, which is a structural protein during enamel bio-mineralization, regulates the shape, size and direction of

mineral crystal growth. The patterns of amelogenin expression in lens fibers, in brain fibers and in nerve trunk fibers suggest a possible role for amelogenin in elongating structures within some developing cells. It is tempting to speculate that the model of elongated amelogenin nanospheres (Du et al., 2005) might be somehow relevant to its role in developing fibers, but other mechanisms are also feasible. In the eye, amelogenin is mainly expressed in the posterior lens epithelium cells that elongate up to several hundred times during the process of differentiation into fiber cells. In the nervous tissues; brain, optic nerve and nerve trunks, amelogenin expression was mostly localized to fiber like structures. Glial and mesenchymal cells are known to surround nerve fibers, whether they are axons or dendrites. Several lines of evidence indicate that amelogenin is mainly expressed by the glial cells; (i) virtually no staining for amelogenin was detected in the cortex that is mainly composed of neuroblasts, whereas the subcortical regions, where many more glial cells exist, was positively stained for amelogenin. (ii) Amelogenin was detected in postnatal glial cells surrounding brain neurons but not in the neurons themselves (Deutsch et al., 2006). A major role of glial cells during development is to support and direct the growth and elongation of neurons.

In 2001, the phenotype of the amelogenin knockout (null) mouse was published. The only phenotype described at that time was of abnormal enamel formation; the teeth of the amelogenin null mouse expressed a hypoplastic enamel phenotype with reduced enamel thickness (Gibson et al., 2001). As often happens, additional, different phenotypes of the amelogenin null mouse were later described. A progressive deterioration of cementum (a mineralized tissue covering the tooth root surface) was observed in the amelogenin null mouse. The defects in cementum were characterized by increased presence of osteoclasts, and were also associated with an increased expression of receptor activator of nuclear factor-κB ligand (RANKL) near the cementum, suggesting that amelogenin may play a role in osteoclastogenesis through the RANKL/RANK mediated pathway (Hatakeyama et al., 2003). In 2006, an additional phenotypic defect was published: the weight of the null mouse was significantly reduced compared with the wild type (Li et al., 2006). No reports have been made on phenotypes in other cranio-facial tissues and body organs. This is not surprising, as only at 2006 amelogenin expression in other mesenchymal mineralizing tissues, such as alveolar bone, long bone and cartilage, and in cranio-facial soft tissues such as brain and salivary gland were published (Deutsch et al., 2006; Haze et al., 2007). Our recent findings of amelogenin expression in active alveolar bone regions (Haze et al. in submission), in epiphyseal growth plate of long bone, its expression in osteoblasts, osteoclasts and osteocytes, as well as the periosteum suggest that amelogenin is active in bone formation and remodeling (Haze et al., 2007). Indeed, as was described above, we found that the recombinant amelogenin brought about regeneration of the tooth supporting tissues via recruitment of mesenchymal stem cells (Haze et al. in submission). In this study it is evident that the expression of amelogenin in different cranio-facial tissues is dynamic and depends on embryonic stage. Our results suggest that additional phenotypes might be revealed when cranio-facial and body tissues in which amelogenin was found to be expressed in the wild type, will be studied in the null mouse. However, it is possible that different compensation pathways lead to normal phenotype in other tissues of the knockout mice.

This study suggests that amelogenin could have structural and signaling roles in the development of various tissues of the cranio-facial complex including the developing tooth germ, and also in other body organs. The results of this study open new horizons as to the function(s) of amelogenin in different tissues during embryonic cranio-facial development. Naturally, there are numerous questions that can be asked and many studies that can be conducted to reveal the molecular mechanisms and pathways with which amelogenin is associated.

MATERIALS AND METHODS

Animals

Embryos from CD-1 mice aged E10.5-E18.5, were used in this study. Embryos from six pregnant mice were used for each age. All experiments were approved by the Animal Care Ethical Committee of the Faculty of Medicine, The Hebrew University of Jerusalem.

Preparation of embryonic tissues

Pregnant mice were sacrificed, the embryos were immediately collected and the heads were removed for further analyses. For histology and immunohisochemistry, the heads were immediately fixed in 4% Para-Formaldehyde

(PFA) over-night at 4°C. The tissues were then washed several times in PBS, and dehydrated in increasing ethanol concentrations of 30, 50, 70, 80, 85, 90, 95 and 100%. The tissues were then immersed in Histoclear/Xylene, embedded in paraffin and frontally sectioned (5 μm) from the nose in an anterior–posterior direction. For mRNA and protein analysis heads were immediately immersed in TRI-REAGENT (MRC, Cincinnati, OH).

RNA isolation and RT-PCR

Total RNA was extracted by homogenizing the cranio-facial complexes of E10.5–E17.5 in TRI-REAGENT (MRC). RNA isolation was performed using the TRI-REAGENT standard protocol. All total RNA extracts were subjected to DNase treatment (DNA free, Ambion, Austin, TX) to eliminate any possible DNA contamination. Total RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was subjected to reverse transcription according to the manufacturer's protocol (Superscript RNase H—Reverse Transcriptase kit, Invitrogene, Carlsbad, CA). RT and PCR amplification were performed using primers designed according to the mouse amelogenin sequence (Gibson et al., 2001), Forward, DD391: 5′-AGA AAC TCA CTG AGC ATA CAC-3′, and reverse, DD390, 5′-GAT GGA GGG ATG TTT GGC TG-3′. PCR products were extracted from 2% agarose gels using Qiaquick gel extraction kit (Qiagen, Valencia, CA) and sequenced at the Center for Genomic Technologies, Hebrew University (Jerusalem, Israel).

Indirect immunohistochemistry

Slides were deparaffinized, hydrated, rinsed in PBS and endogenous peroxidase activity was blocked by 3% H_2O_2 (diluted in methanol) for 10 min. Slides were blocked in nonimmune goat serum for 20 min (Histostain-SP kit, Zymed laboratories Inc., San Francisco, CA), followed by over-night incubation of the primary antibody (diluted in PBS) at 4°C in a humidified chamber. The first antibodies used were: (a) 270 Polyclonal rabbit antibody, raised against amelogenin N-terminus (MPLPPHPG) (identical in mouse, human, etc.) and diluted in PBS to 1/500–1/1000. (b) 859 polyclonal rabbit antibody raised against rat amelogenin. The specificity of the antibodies was previously determined (Haze et al. in submission), by subjecting the antibodies to sections obtained from the amelogenin knockout mouse (Gibson et al., 2001) mandible. No staining for amelogenin was obtained in the mandible, including, among others, the ameloblasts and enamel. On the other hand, using the same conditions, some of the corresponding wild type tissues, including the ameloblasts and enamel showed strong staining for amelogenin.

After rinsing, slides were treated according to the Histostain-SP kit protocol (Zymed laboratories inc.). Negative controls included PBS in place of the first antibody. For better viewing of the histology of the tissue, the slides were stained with hematoxylin (Pioneer Research Chemicals, Colchester, Essex blue staining). All slides were examined by Axioskop (Zeiss, Göttingen, Germany). The pictures were taken using Coolpix 990 digital camera (Nikon, Tokyo, Japan), and ProgRes C10 (Jenoptik, Jena, Germany).

Preparation and culturing of mouse embryonic mandibles

Freshly harvested (13.5 day) embryo heads were placed in Dulbeco's modified Eagle's medium (DMEM) (with L-glutamatmine), containing 20 units/mL penicillin/streptomycin (Biological Industries, Beit Ha'emeck, Israel; Sigma-Aldrich, Rehovot,Israel). The mandibles were dissected from the cranio-facial complex by using watchmaker's forceps (FST, Vancouver, Canada) and 27-gauge sterile needles (Sherwood Medical Industries, Athy, Ireland). The dissected mandibles were then placed on a 0.1 μm Millipore filter (Millipore Southampton) coated with Matrigel (BD Biosciences, San Jose, CA), and then covered with Matrigel. The filter paper with the mandible was then placed on a 35 mm triangle stainless steel metal grid, with 0.25 mm diameter mesh wire (Goodfellows, Cambridge), placed in Falcon 60 mm Center-well Organ Culture dishes (BD Biosciencse, San Jose, CA). The center well was filled with DMEM (with L-glutamatmine) and 20 u/mL penicillin/streptomycin, up to the mesh surface, and the narrow well, surrounding the center well, was filled with double distilled sterile water. The cultures were grown in a standard incubator at 37°C with an atmosphere of 5% CO_2. After incubation in culture for one day, explants were washed in ice-cold methanol for 2 min and then fixed in 4% paraformaldehyde for 2 hr at room temperature. The explants were then embedded in paraffin, sectioned and counterstained with eosin for histology.

Bead preparation and placement on mouse embryonic mandible explant cultures

Affi-gel agarose beads (Bio-Rad Laboratories, Hercules, CA) (75–100 μm) were separated by size under stereomicroscope, washed thoroughly in PBS, air dried and suspended in rHAM^{+} (Taylor et al., 2006) (1 μg/μL) dissolved in 0.05 M acetic acid solution, at 37°C for 30 min. Before use, beads were washed in PBS (neutralizing the acid), and the beads were then placed on E13.5 mandibular explant, in the incisor region, the molar region and on the mandible diastema region (experimental beads). Control beads were prepared in a similar manner by using only PBS, and were placed on the contra-lateral side of the mandible in similar regions as the experimental beads. Mouse embryonic mandible explant cultures containing Affi-gel beads were grown for one day, as described below

Preparation of 250 μm thick frontal sections of mouse embryonic mandibles for DiI studies

E13.5 and E14.5 mouse embryonic mandibles were frontally dissected into 250 μm thick sections, using Mcllwain Tissue Chopper (Science Products GmbH, Hofheim, Germany) as described in Matalova et al., 2005. Briefly, sections containing first molars, or incisors, were selected under the microscope in medium. Affi-gel beads (control and experimental) were placed on the tooth germ mesenchymal region, and CellTracker^{TM} CM-DiI (Molecular probes, Invitrogen, Eugene, OR) was injected into the mesenchymal cells in the vicinity of the beads using a mouth pipette. Slices were placed on a 0.4 μm FalconTM cell culture inserts (Falcon, BD Labware, Franklin Lakes,NJ). The sliced tissue was then covered with Matrigel (BD Biosciences), and cultured as was described above for the mandibular explant for up to 3 days. The cultures were photographed every 24 hr using a Leica dissecting microscope under either bright field, dark field or both (for superimposition) to monitor the DiI at 0, 1 and 2 days. Cultures were fixed at day 2 and embedded in wax for sectioning. After sectioning the localization of DiI was photographed using a Zeiss Fluorescence compound microscope. Sections were then stained for histology and re-photographed. The DiI label is fixable but lost after staining for histology.

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